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Mouse mammary cell lines containing either wild type or mutant p53 were used to examine distinct p53 forms following treatment with the DNA damaging agents actinomycin D and genistein using immunofluorescence, sequence-specific DNA binding, or western immunoblotting. We show that p53 genotype does not predict p53 response to DNA damage or function. For example, cell lines containing wild type p53 had one of the following three disparate "molecular phenotypes": 1) p53 expression (TM9) is not induced by genistein treatment and the protein is unable to bind DNA, 2) p53 expression (TM10) is induced by genistein treatment and both active and latent forms are present for DNA binding, 3) only latent forms (TM12) are available for DNA binding that are not induced by genistein treatment. This variability of induction and activity may depend on p53 associated factors. To examine this possibility, we have isolated mammary specific p53-associated proteins, subjected them to microsequencing and identified them as three splicing factors and two ribosomal proteins. The DNA binding sequence of the endogenous active form of mouse p53, p53as, has been shown to contain the same hallmarks as that for p53. In addition to the p53 consensus sequence, non-consensus DNA sequences have been confirmed and novel gene targets of p53 suggested.

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M. Kurusz-Martin (JM) 9/28/98

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## INTRODUCTION

Breast cancer results from changes in any one of several genes, however, one of the most frequent genetic alterations found is in the tumor suppresser, p53. p53 functions as a protector of genomic integrity by initiating either cell cycle arrest or apoptosis in response to cellular distress or DNA damage. Thus, mutation of the p53 protein can lead to accumulation of DNA damage eventually giving rise to cancer.

p53 protein exists in multiple forms in mouse and human cells (1) and is often found as a latent form which requires activation in order to bind sequence-specific DNA (2). This laboratory has identified an alternatively spliced p53 protein, p53as, in normal mouse cells and tissues which is constitutively active for sequence-specific DNA binding. Studies of p53as are being done to provide leads abut active and latent forms of p53 protein in human cells, where activated forms are still largely uncharacterized. p53as protein contains 17 different amino acids and is 9 amino acids shorter at the C terminus than p53. These modifications convey differences in p53as characteristics compared to p53. p53as is preferentially present in G2 phase cells whereas p53 is detectable in both G1 and G2 cells (3). p53as responds with different kinetics to DNA damage caused by genistein (1). In addition, p53as does not require activation to bind sequence specific DNA. On the other hand, both p53 and p53as function as suppressers of cell growth (1) and bind the same DNA sequences (4).

The experiments set forth in this proposal sought to examine the role p53 and p53as play in mammary carcinogenesis and the progression of mammary cancer. A murine breast cancer model (5;6) was used that permitted examination of p53 and p53as at various stages of preneoplasia and malignancy. The Specific Aims of this proposal

were: 1) to examine the expression of p53 and p53as during mammary cancer development, 2) to determine the half life of p53 and p53as proteins and to detect associated proteins, and 3) to determine the oligomerization properties and DNA binding sequence specificity of p53as.

## METHODS

### TASK 1 (Aim 1) Determination of p53 and p53as expression in mouse mammary model

Immunofluorescence assays were carried out on 5 x $10^4$ cells plated on coverslips. Cells were grown until 50% confluent and then treated for 48-72 hours with actinomycin D (0.5 nM), genistein (60 μM), X-ray (2000 rads), or left untreated followed by ethanol fixation. Primary antibodies specific for p53 or p53as or antibodies able to differentiate between wild type and mutant conformation of p53/p53as were incubated with the coverslips for 1 hour followed by incubation with Texas Red-conjugated goat anti-rabbit or FITC-conjugated goat anti-mouse secondary antibodies for 30 min. Coverslips were mounted on microscope slides and examined by fluorescent microscopy.

Reverse transcription-polymerase chain reaction (RT-PCR) assays were carried out on RNA isolated from TM cells grown to 80% confluency. Total RNA was isolated using Ultraspec RNA solution (Biotecx Laboratories, Inc.) and an aliquot used in an RT assay containing AMV-reverse transcriptase (Promega). An aliquot of the RT assay was then used in PCR containing primers for amplification of p53 or p53as and Taq polymerase (Boehringer Mannheim).

Gel shift assays to identify the functional forms of p53 in TM cells were carried out using cell lysates obtained from untreated, genistein (20-30 μM) treated, or X-ray (400 rads) treated cells. Approximately 25 μg of total protein was added to 15 μl gel shift reactions containing 1 μg of poly[d(I-C)], binding buffer, BSA, and $^{32}$P-end-labeled p53 DNA consensus sequence probe. Antibodies specific for p53 or p53as were added to some reactions. Reactions were separated on 4% nondenaturing polyacrylamide gels and visualized by autoradiography.

**TASK 2 (Aim 2,3) Production and purification of p53as and p53 proteins in the baculovirus system**

p53 and p53as were initially cloned into a baculovirus shuttle vector (pVL1393) and transfected into Sf9 cells. High titer virus was obtained and optimal protein production following infection determined. Antibody affinity columns were used to purify p53 and p53as, however, protein yields were very low. More recently, p53 and p53as have been cloned into histidine-tagged baculovirus shuttle vectors (pFastBac HT) which allows for efficient and easy protein purification. Both p53 and p53as purified proteins are available in the laboratory.

**TASK 3 (Aim 2,3) Determination of oligomerization properties of p53as**

Radioactive *in vitro* translated p53 or p53as was fractionated by gel filtration on a Superose 6 column (Pharmacia) by FPLC. Fractions were analyzed by SDS-PAGE and visualized by autoradiography. The positions of monomers, dimers, and tetramers of p53 or p53as were determined by comparison with molecular weight standards.

**TASK 4 (Aim 2) Determination of the half-life of p53as and p53 proteins**

An asynchronous half life of p53 or p53as was determined by growing TM cells in the presence of 0.5 mCi $^{35}$S-cysteine and $^{35}$S-methionine/ml of medium for 1 hour followed by growth in media without radioactivity for various time points. Cells were harvested, lysed, and antibodies specific for p53 or p53as were added to cell lysates and gently agitated at 4°C overnight. Protein A sepharose was then added to the mixture and agitation continued for 2 hours. Immune complexes were collected by centrifugation, washed 3 times, and resuspended in Laemmli loading buffer for SDS-PAGE analysis. Proteins were visualized by autoradiography.

**TASK 5 (Aim 3) Determination of a p53as-specific DNA binding site(s) and assay of p53as and p53 binding activities**

p53as-specific DNA binding sequences were identified using cyclic amplification and selection of targets (CASTing) (7-9). A 96-basepair DNA probe was used which contained 60 random nucleotides flanked by 18 basepair primer sites. *In vitro* translated p53as was allowed to bind the probe DNA and protein-DNA complexes isolated using magnetic beads bound to a p53as-specific antibody and a magnet. The bound DNA probes were amplified by PCR, cloned, and sequenced. Sequences were analyzed for common motifs and tested for binding by p53as and p53 in DNA binding assays. Motifs identified in the p53as binding sequences were used in FASTA searches. Additional probes were also designed and synthesized to test the ability of p53 or p53as to bind variations of the sequences identified by CASTing.

**TASK 6 (Aim 2) Comparison between p53as- and p53-associated proteins**

Plasmids containing full length p53, p53as or approximately 100 amino acids of either C terminus were constructed as glutathione-s-transferase (GST) fusion proteins in the pGEX-2TK expression vector. Fusion proteins were produced in bacteria by induction with 200 mM isopropyl-D-thiogalactopyranoside (IPTG). Following cell harvesting and lysis, cell extracts were added to glutathione beads and the mixture slowly agitated for 2 hours at 4°C. Beads were then washed 5 times to remove unbound proteins and stored at 4°C overnight.

TM10 nuclear extracts were prepared by harvesting cells and adding 5 packed cell volumes (PCV) of hypotonic lysis buffer (10 mM Hepes, pH 7.9/75 mM spermidine/0.15 mM spermine/0.1 mM EDTA/0.1 mM EGTA/10 mM KCl/1 mM DTT) to pellet. After 10 min on ice, the cells were pelleted by centrifugation in a table top centrifuge followed by lysis in a Dounce homogenizer in 2 PCV of the above buffer. Nucleii were pelleted by centrifugation at 12,000 rpm in an SS34 rotor followed by resuspension in 140 μl/$10^8$ cells of 20 mM Hepes, pH 7.9/0.75 mM spermidine/0.15 spermine/0.2 mM EDTA/2 mM EGTA/2 mM DTT/20% glycerol. Aliquots of 5 M NaCl were added to equal 70 μl of 5 M NaCl/900 μl of resuspended cells and the mixture slowly agitated at 4°C for 40 min followed by centrifugation in a swinging bucket rotor at 50,000 rpm for 30 min at 4°C. PMSF and DTT were added to 1 mM and 5 mM, respectively, to the supernatant and the mixture dialyzed against 10 mM Tris-HCl, pH 8.0/100 mM NaCl/10 mM $MgCl_2$/1 mM EDTA/10% glycerol for 3 hours. Protein concentration was determined by a Bradford assay and nuclear extracts stored at -80°C.

TM10 nuclear extract was added to the prepared beads at ~2.5 μg of protein/1 PCV of bead and incubated on ice for 1 hour. Beads were harvested by centrifugation at 1000 rpm for 1 min, washed 4 times in 1 PCV of 10 mM Tris-HCl, pH 8.0/10 mM $MgCl_2$/1 mM EDTA/10% glycerol/100 mM NaCl, and associated proteins batch eluted in 1 PCV of the same buffer with increasing amounts of NaCl (200, 500, and 1000 mM). Aliquots of eluted fractions were analyzed on 7.5% and 12% SDS-PAGE and the proteins compared to proteins associated with beads containing GST protein alone.

Fractions were concentrated by using a Micron 10 concentrator (Amicon) and proteins separated by SDS-PAGE. Gels were stained with Coomassie Blue and protein bands of interest excised for analysis by the HHMI Biopolymer/W.M.Keck Foundation Biotechnology Resource Laboratory at Yale University.

Molecular subcloning of hnRNP A2 and CArG into a baculovirus vector (pFastBac HTc) was carried out using PCR to generate cDNA with compatible restriction sites from template cDNA obtained from Dr. T. Miwa, Osaka University (CArG box-binding factor) (10) and Dr.G. Dreyfuss, U of Pennsylvania Medical School (hnRNP A2) (11). Both cDNAs were generated with 5' EcoRI sites to place the ATG in frame in the baculovirus expression vector. The 3' cloning sites used were XbaI for CArG and KpnI for hnRNP A2. DNA sequencing has been performed by the RPCI Biopolymer Laboratory on the hnRNP A2 clone to verify the reading frame and will also be carried out on the CArG clone when obtained.

## RESULTS AND DISCUSSION

### TASK 1 (Aim 1) Determination of p53 and p53as expression in the mouse mammary model

As previously reported, RT-PCR analyses indicated that all TM lines expressed mRNA for both p53 and p53as (1995 Report, Figure 2; 1996 Report, Figure 1). Immunofluorescence studies determined the presence of p53 and p53as proteins in all TM cell lines (1995 Report, Figure 1 and Table 2). In addition, immunohistochemistry analysis of TM3 (weakly tumorigenic) and TM10 (weakly tumorigenic) tumor tissue indicated expression of p53 and p53as proteins (1996 Report, Table 1). Table 1 included in this report shows the genotypes and tumor phenotypes of the TM lines used in this study. A summary of the immunofluorescence data is shown in Table 2 and includes the inducibility of p53 or p53as by actinomycin D or genistein, two DNA damaging agents. These experiments were designed to determine the feasibility of using a DNA damaging agent to enhance visualization of p53 and p53as for protein stability investigations. As Table 2 shows p53 in TM3 (mutant) expression is increased by actinomycin D treatment and p53 in TM10 (wild type) expression is induced by genistein treatment. The expression of p53 in the other TM cells is unaffected by either treatment.

As reported last year, further experiments examining p53 or p53as sequence specific DNA binding function following genistein treatment in TM3, TM4, TM9, and TM10 resulted in two interesting observations. First, only TM4 (mutant p53) and TM10 (wild type p53) had p53 protein forms that were able to bind DNA (1997 Report, Figures 1 and 2). Although TM3 (mutant p53) and TM9 (wild type p53) cells did contain p53 and p53as proteins as indicated by immunoblot analyses (1997 Report, Figure 6), neither

protein was able to bind DNA (1997 Report, Figure 3). Second, in TM4, neither p53 protein expression nor DNA binding ability was induced by genistein treatment.

Figure 1 shows the DNA binding assays utilizing TM10 lysates. The pattern of bands is similar to those seen using mouse epidermal cell lysates (1). As indicated in Figure 1, these bands include (1) latent p53/p53as hetero-oligomers, (2) latent p53/p53as hetero-oligomer, (3) active p53as homo-oligomers, and (4) active p53/p53as hetero-oligomer. Quantitaton indicates peak binding activity occurs at 3 hours following treatment and is reproducibly two fold higher than basal levels. In addition, immunoblots of TM10 lysates shows that protein expression is also induced approximately two fold at 3 hours following genistein treatment (Figure 2).

We have extended this study by examining the TM12 (island) cell line, which contains wild type p53, for sequence specific DNA binding following treatment with genistein. Figure 3 shows the results of one such experiment and indicates that wild type latent p53 protein is able to bind DNA following activation by PAb421 (forms 1 and 2) unlike wild type p53 isolated from TM9 cells. However, DNA binding function is not induced by genistein treatment in TM12, unlike the wild type p53 in TM10 cells. In addition, TM12 cells appear to contain no active forms of p53 (forms 3 and 4).

We also examined the effect of X-ray treatment on p53 and p53as DNA binding function in TM10 cells. Figure 4 shows the results of these experiments and indicates that X-ray induction is not as well defined as genistein induction is in TM10 cells. Although there is some induction by X-ray at 3 hours following treatment, the increased expression is sustained through 24 hours unlike the induction seen with genistein.

**TASK 2 (Aim 2,3) Production and purification of p53as and p53 proteins in the baculovirus system.**

Purified proteins were anticipated to be needed to determine the oligomerization properties of p53as (Task 3) and as an alternative means to identify p53-associated proteins (Task 6). Therefore, purified baculovirus produced recombinant p53 and p53as proteins have been produced and are available in the laboratory. In addition to these, *in vitro* translated proteins have been used to establish the oligomerization properties of p53as, and GST/p53-fusion proteins have been used to isolate p53-associated proteins.

**TASK 3 (Aim 2, 3) Determination of oligomerization properties of p53as**

The task was completed and reported in the first year progress report (1995 Report, Figure 8). Both p53 and p53as form homo- and hetero-tetramers.

**TASK 4 (Aim 2) Determination of the half life of p53as and p53 proteins**

The half life of p53 in asynchronous TM3 and TM4 cells was determined to be approximately 5 hours, while in the same cells the half life of p53as is approximately 6 hours (1995 Report, Figures 14-17). The relatively long half life reflects that these cells contain a mutated p53 which exhibits increased stability when compared to wild type p53. In normal fibroblasts, p53 is reported to have a 30 minute half life (12), while in normal human mammary epithelial cells, the half life of p53 has been observed to be 3 hours (13).

We have tried different methods to synchronize TM cells in order to determine the half life of p53 and p53as in the various cell cycle phases. Using centrifugal elutriation, separation of S phase from G2/M phase was not achieved (1995 Report, Figures 9 and 10). Density arrest and/or serum starvation produced a large fraction of non-cycling G1

cells at all points of the cell cycle (1996 Report, Figures 2 and 3). Experiments utilizing the G1 arrest agent, aphidicolin, also gave a significant background of non-cycling G1 cells (1997 Report, Figures 7 and 8). Thus, it has not been possible to determine the cell cycle dependence of half life of p53 and p53as in the mouse mammary epithelial cells.

**TASK 5 (Aim 3) Determination of a p53as-specific DNA binding site(s) and assay of p53as and p53 binding activities**

The p53as DNA binding sequences isolated by CASTing (see Methods) contained certain hallmarks of the p53 consensus sequence (14). Sequences of 14 sequences that bind p53as are shown in Table 3. Three of the sequences were duplicates, e.g. 24 and 28, giving rise to 11 unique p53as binding DNA sequences. All of the sequences contained at least one CATG motif and the majority (9/11) contained 2 or more copies of this motif. Only two of the sequences, 13 and 38/64, fit the consensus requirement of 3 purines upstream and 3 pyrimidines downstream of the CATG motif. The remaining 9 sequences deviated from the consensus sequence in this respect.

Small regions of homology to previously isolated p53 DNA binding sequences were also noted and are shown in Table 4. Included in this table are the published sequences in which the p53 DNA binding sequences are found. In addition, there was no difference in the binding abilities of p53 and p53as. Both proteins bound all of the isolated sequences (4). FASTA searches of GenBank with segments of the isolated sequences indicated several genes that contained the segments in promoter or intron regions. The rat mdr1 and mouse thrombospondin genes were two such genes, both of which have been shown to be regulated in humans by p53 (15;16).

**TASK 6 (Aim 2) Comparison between p53as- and p53-associated proteins**

Last year we reported that TM10 nuclear extracts contained proteins that bound to GST-p53 and GST-p53as fusion proteins but not to GST protein alone (1997 Report, Figures 10-12). We have now scaled up this protocol, making nuclear extract from 4 x $10^9$ TM10 cells and allowing this nuclear extract to associate with 1 ml of prepared glutathione-GST-fusion protein bead slurry. Figure 5 shows a Coomassie Blue stained 7.5% polyacrylamide gel on which an aliquot of the GST-fusion proteins have been resolved before allowing association with nuclear extract to occur. The proteins of interest are indicated with arrows. Varying amounts of BSA have also been placed on the gel to allow quantitation of the GST-fusion proteins. Figure 6 shows a silver stained 12% polyacrylamide gel containing aliquots of the 500 mM and 1M NaCl eluted fractions following association of TM10 nuclear extract with the indicated GST-fusion proteins. GST protein alone has brought down very few proteins, while p53, p53 C terminus, p53as, and p53as C terminus contain many associated proteins. Two associated proteins that differ between p53 and p53as (approximately 40 and 42 kD) are indicated by asterisks. Several associated proteins were also present in fractions obtained from different nuclear extracts in other experiments. For example, a 100 kD protein that was not associated with GST-fusion protein was found in all preparations associated with both p53 and p53as. p53as and p53as-c terminus did not associate with as many proteins, especially lower molecular weight proteins, as p53 and p53-c terminus proteins did. This may reflect the loss of c terminal amino acids involved in protein association.

The fractions from Figure 6 were concentrated using a Micron 10 filter (Amicon), resolved by 12% SDS-PAGE, and stained with Coomassie Blue. The

concentration resulted in the loss of several bands including the 40 and 42 kD bands specific for p53. This loss may have been due to selective binding of some proteins to the concentrating filter membrane. Concentrated proteins were separated by gel electrophoresis and several bands of interest were excised and sent for analysis to the HHMI Biopolymer/W.M.Keck Foundation Biotechnology Resource Laboratory at Yale University. Table 5 shows the results of these analyses. The sample names are based on the approximate molecular weight of each isolated protein determined by molecular weight markers on the same gel from which the bands were excised. One band (p16) contained more than one protein and therefore could not be sequenced. Two of the proteins share homology with E. coli ribosomal proteins (S4 and L2) while the remaining three proteins are involved in splicing (hnRNP A2/B1, hnRNP A3, and CArG). Two of the proteins, CArG and hnRNP A3, have also been isolated using the same GST-pull down method with mouse epithelial cell nuclear extract (A. Amador and M. Kulesz-Martin, unpublished).

We have obtained molecular clones of CArG (10) and hnRNP A2 (11) to subclone these genes into pFastBac vectors for baculovirus expression. So far, hnRNP A2 has been subcloned into pFastBac HTc using a product generated by PCR and the reading frame verified by sequencing. This will now be used to produce purified hnRNP A2 protein to verify the association with p53 by immunoprecipitation or other experiments. PCR amplification of CArG using primers incorporating restriction sites for cloning into pFastBac HTc has been optimized using the Optiprime Kit (Stratagene). Figure 7A shows the first step in this optimization with every lane representing a different PCR buffer. Three of the buffers (1, 5, and 9) that resulted in the best yield of

expected product (885 bp) were chosen for further examination. PCR reactions were carried out containing one of these buffers plus the additives indicated in Figure 7B. Buffers 5 and 9 with the additive formamide or DMSO showed the best amplification of the 885 bp fragment. Buffer 9 with formamide will be used for amplification for cloning.

## CONCLUSIONS

### Task 1 Determination of p53 and p53as expression in the mouse mammary model

This task has been completed and shows that the mammary cell lines TM3, TM4, TM9, TM10, and TM12 all express p53 and p53as mRNAs and proteins. Immunohistochemistry analysis of two tumors from weakly tumorigenic cell lines, TM3 and TM10, indicate both tumors contain p53 and p53as proteins. Treatment of the TM cell lines with the DNA damaging agent, genistein, followed by analysis of p53 sequence specific DNA binding activity in these cells was examined. TM3 (mutant p53) and TM9 (wild type p53) contained p53 and p53as proteins as shown by immunoblot. However, none of the p53 proteins produced in these cells was able to bind DNA. TM4 (mutant p53) did contain p53 protein that could bind DNA, but the protein expression and DNA binding function was not induced by genistein treatment. In TM10 (wild type p53), all p53 forms bound sequence specific DNA and both protein expression and binding activity were induced by genistein treatment. In addition, TM12 cells (wild type p53) only p53 latent forms were present that were activated by PAb421 and DNA binding ability was not induced by genistein treatment. Thus, the three cell lines, TM9, TM10, and TM12, that contain wild type p53 show three different sequence-specific DNA binding capabilities: (1) in TM9, neither active nor latent forms bind DNA or are induced by genistein treatment; (2) in TM12, only latent forms bind DNA and there is no

induction by genistein treatment; and (3) in TM10, both latent and active forms of p53 bind DNA and are induced maximally at 3 hours following genistein treatment. This implies that p53 has "molecular phenotypes" that may depend on the associated protein environment within the cell.

These data indicate that p53 genotype does not predict p53 function and have ramifications in cancer therapy. Studies of chemotherapy treatment and p53 genotype has shown both a positive correlation (17;18) and a lack of correlation (19-21)between the two variables in predicting outcome. Re-examination of treatment response and p53 function for a correlation may indicate p53 function could serve as a biological marker to predict treatment response. We have recently submitted proposals to begin to examine the hypothesis that p53 function better predicts prognosis and response to chemotherapy that p53 genotype. The purpose of the first proposal is to develop a procedure for assessment of p53 functional status in breast tumors. This would be accomplished by looking for changes in the signal of injected signal-tagged p53-specific DNA probes by magnetic resonance imaging (MRI) or positron emission tomography (PET). In the second proposal, we plan to examine the induction of p53 active and latent forms by treatment of human breast tumor grown in SCID mice with radiation, tamoxifen, adriamycin, methotrexate, and genistein. The current project results suggest that active forms in human cells will be recognized by sequence-specific DNA binding in the absence of antibody PAb421, and by specific antibodies for active forms in human cells when they become available. The presence of active p53 forms will predict a positive response to these therapeutic agents.

**Task 2 Production and purification of p53as and p53 proteins in the baculovirus system**

This task has been completed and purified proteins are available in the laboratory for future co-immunoprecipitation experiments as discussed in Task 6.

**Task 3 Determination of oligomerization properties of p53as**

This task has been completed and indicates that both p53 and p53as form homo- and heterodimers and tetramers. p53as is constitutively active for DNA binding while p53 requires activation and some heterotetramers acquire the characteristics of p53 for DNA binding. Thus, regulation of the ratio of p53 and p53as may be the means of varying the functions of p53 and p53as.

**Task 4 Determination of the half life of p53as and p53 proteins**

Determination of the half life of p53 and p53as in asynchronous TM3 or TM4 cells has been completed. The half life of p53 is approximately 5 hours and of p53as approximately 6 hours. However, obtaining TM cells in various cell cycle phases to establish the half life of p53 and p53as in G1, S, and G2/M has been unsuccessful. Centrifugal elutriation, serum starvation, and aphidicolin-induced G1 arrest have all been tried. Centrifugal elutriation did not reproducibly separate S from G2/M cells. G1 arrest using serum starvation or aphidicolin gave high backgrounds of noncycling G1 cells upon release of the G1 arrest. These results may reflect a normal characteristic of epithelial cells in which a state of equilibrium is achieved between cycling and non-cycling cells. In order to determine cell cycle specificity of p53 forms, more readily synchronized cells, not breast cells, may have to be used.

**Task 5 Determination of a p53as-specific DNA binding site and assay of p53as and p53 binding activities**

This task has been completed. The sequences retrieved using CASTing (see Methods) with p53as contain characteristics of the p53 consensus DNA sequence (14). Both p53 and p53as bind the same DNA sequences. However, CASTing results indicated p53 specific binding to several non-consensus motifs. A FASTA search revealed a sequence in the rat mdr1 promoter with 100% homology to an isolated CASTing sequence. Recently, this same rat promoter sequence was shown to be p53-responsive and essential for basal and daunorubicin-inducible promoter activity (22). Another homologous sequence in the thrombospondin gene has been identified for future study. These non-consensus sequences may reveal additional downstream transcription target genes of p53.

**Task 6 Comparison between p53as- and p53-associated proteins**

Five p53-associated proteins from TM10 nuclear extracts have been isolated and identified. Two are homologues of E. coli ribosomal proteins, S4 and L2. p53 has been shown to complex with another ribosomal protein, L5, when in a complex with mdm-2 (23). In addition, a small amount of cellular p53 exists covalently bound to 5.8S RNA (24) which also can complex in solution with 5S RNA and L5 ribosomal protein (25).

Three other p53-associated proteins isolated from TM10 are splicing factors, hnRNP A3, hnRNP A2/B1, and CArG binding factor. CArG binding factor and hnRNP A3 have also been isolated independently from mouse epithelial cell nuclear extracts in our laboratory. hnRNP A2 cDNA has been subcloned into a baculovirus vector for protein expression and CArG cDNA is in the process of being cloned in the same vector.

Future studies will verify p53 association by examination of GST-hnRNP A2 or -CArG interaction with *in vitro* translated p53. Co-localization of p53 and hnRNP A2 or CArG will also be examined in cells by immunofluorescence. The effect of hnRNP A2 or CArG on p53 sequence-specific DNA binding will also be determined.

The association of p53 with splicing factors in cells has not been examined. If p53 associates with splicing factors *in vivo*, then the p53 response to DNA damage may play a role in splicing, either by increasing or decreasing total splicing and thus the amounts of mRNAs, or by altering the availability of splice variants and thus specific mRNAs. In addition, mutation of p53 may have different capabilities contributing to a novel mechanism of carcinogenesis.

| LINE | GENOTYPE | PHENOTYPE |
|---|---|---|
| TM3 | Mutant<br>One allele, insertion $Ser^{233-234}$<br>No differing allele | Weakly tumorigenic |
| TM4 | Mutant<br>One allele, deletion of amino acids 123-129<br>One allele, Cys --> $Trp^{138}$ | Highly tumorigenic |
| TM9 | Wild type | Weakly tumorigenic |
| TM10 | Not sequenced | Weakly tumorigenic |
| TM12 | Wild type | Weakly tumorigenic |

Table 1. The genotype and tumor phenotype of TM cell lines are indicated. Numberical superscripts indicate amino acid positions of mutation in mouse p53 protein (390 amino acids).

| Cell Line | p53 Reactivity | p53as Reactivity | p53 Inducibility Actinomycin D | p53 Inducibility Genistein | p53 Inducibility X-ray | 53as Inducibility Actinomycin D | p53as Inducibility Genistein | p53as Inducibility X-ray |
|---|---|---|---|---|---|---|---|---|
| TM3 | + | + | ++ | + | nd | ++ | + | nd |
| TM4 | + | + | + | + | + | + | + | + |
| TM9 | + | + | nd | + | nd | nd | + | nd |
| TM10 | + | + | + | ++ | + | + | ++ | + |
| TM12 | + | + | + | + | nd | + | + | nd |

Table 2. Immunofluorescence reactivity of p53 and p53as antibodies in TM cell lines.

6 ATGGGGG**CATG**AGATTAAATAACCCAAATGAGGG**CATG**CGC**CATG**TCTGGCCC

13 AGCGTCAACAAGCGATTAGACACACGAA**CATG**TCCGGA**CATG**TTCTAGCAAGAGCGTCT

14 ATGGGGG**CATG**AGATTAATAACCCAAATGAGGGCATCCG**CATG**CATGCCTCGGCCC

18 CCACCGGGA**CATG**TCGTTCTTGTTTTTCTGAATGGACTTGTTCGCATAAAGAGCCTGG

21 GCCAACGAGTGAACGGGCATCCG**CATG**TTAACGGTTTTGGCTTATCGCTGTGAT

24/28 TATCACAGCGATAAGCCAAACCGATTAA**CATG**CG**CATG**CCGGTTCACTCTTGGCGG (54N PRIMER)

CAGAAGACTGCCCGGG**CATG**TTCTACCAAACTGTTCCCCTTCCTCCTGTG

36 GCCAACGAGTGAACGGG**CATG**CGCATGTTAACGGA (18N PRIMER)
GCCACGGACCTGATCACGGGTCTGTCTAAAGCGGTTTGAATCGT**CATG**GGTGAATGGTAA

42/59 GCCAACGAGTGAACGGG**CATG**GCG**CATG**TTAACGGTTTTGGCTTATCGCTGTGGT

62 ACGTGTAA**CATG**CCCGGGATGTTGTTCGAGCCCGAAGAGTCAAAGGGTTGAGACTAGGC

63 A**CATG**AACAGGTTTAAGGTTAATGA**CATG**CCCGGG**CATG**CCGCGGTCTTTCAGTAGGGT

38/64 AGCCGCTCTTGCTAGAA**CATG**TCCGGA**CATG**TTCGTGTGTCTATCGCTTGTTGACGCT

Table 3. p53as-binding sequences retrieved by CASTing.

Sixty four transformants containing PCR products obtained from CASTing were examined for DNA binding by *in vitro* translated p53as. The fourteen positive molecular clones were sequenced and the sequences are shown above. Some of the molecular clones were duplicates (e.g. 24/28) and most likely represent ligation of the same PCR amplification product.

| Motif | Casting Sequences | Published Sequences | References |
|---|---|---|---|
| PuPuPuCA/TT/AGPyPyPy | 13, 64/38 | | 14, 26 |
| GAACATGTCCGGACATGTTC | 13, 64/38 | | |
| CCCGGG | 24/28, 62, 63 | Cyclin G, p53con | 27, 26 |
| TTGGC* | 21, 24/28, 42/59 (inverted repeats) and 36 | MgBH6 | 28 |
| CTTG | 18, 24/28, 38/64) | cyclin G, RGC, MgBH5 | 27, 29, 28 |
| ACTTG | 18, 24/28 | RGC, MgBH6 | 29, 28 |
| TGGCT | 21, 42/59 | MgBH6 | 28 |
| TGCCT | 14 | RGC, 50-2 | 29, 30 |
| TGCCC | 24/2, 62, 63 | cyclin G, p53con | 27, 26 |
| TGTCC | 13, 38/64 | WAF-1, p53con | 14, 26 |
| GGCAAGCCT | none | cyclin G, 50-2 | 27, 30 |

Table 4. Summary of motifs found in CASTing sequences and published p53-binding sequences. CASTing sequences are numbered as explained in Table 3. *Extended to TTGGCT in 21, 42/59, and MgBH6

| Sample | Protein Identification |
|---|---|
| p16 | inconclusive (a mixture of 2 protein) |
| p25 | S4 ribosomal protein (E. coli) |
| p32 | L2 ribosomal protein (E. coli) |
| p32-S | hnRNP A2/B1 |
| p38 | hnRNP A3 |
| p40 | CArG binding factor, mouse |
| p45 | An unidentified hnRNP (but not hnRNP A3) |

Table 5. p53-associated proteins isolated from TM10 mammary cells nuclear extract. Selected proteins similar to those shown in Figure 6 were excised from a polyacrylamide gel and analyzed by MALDI mass spectroscopy and/or microsequencing in the W.M. Keck Foundation Biotechnology Resource Laboratory at Yale Uiversity. Results were compared to EMBL and ProFound databases.

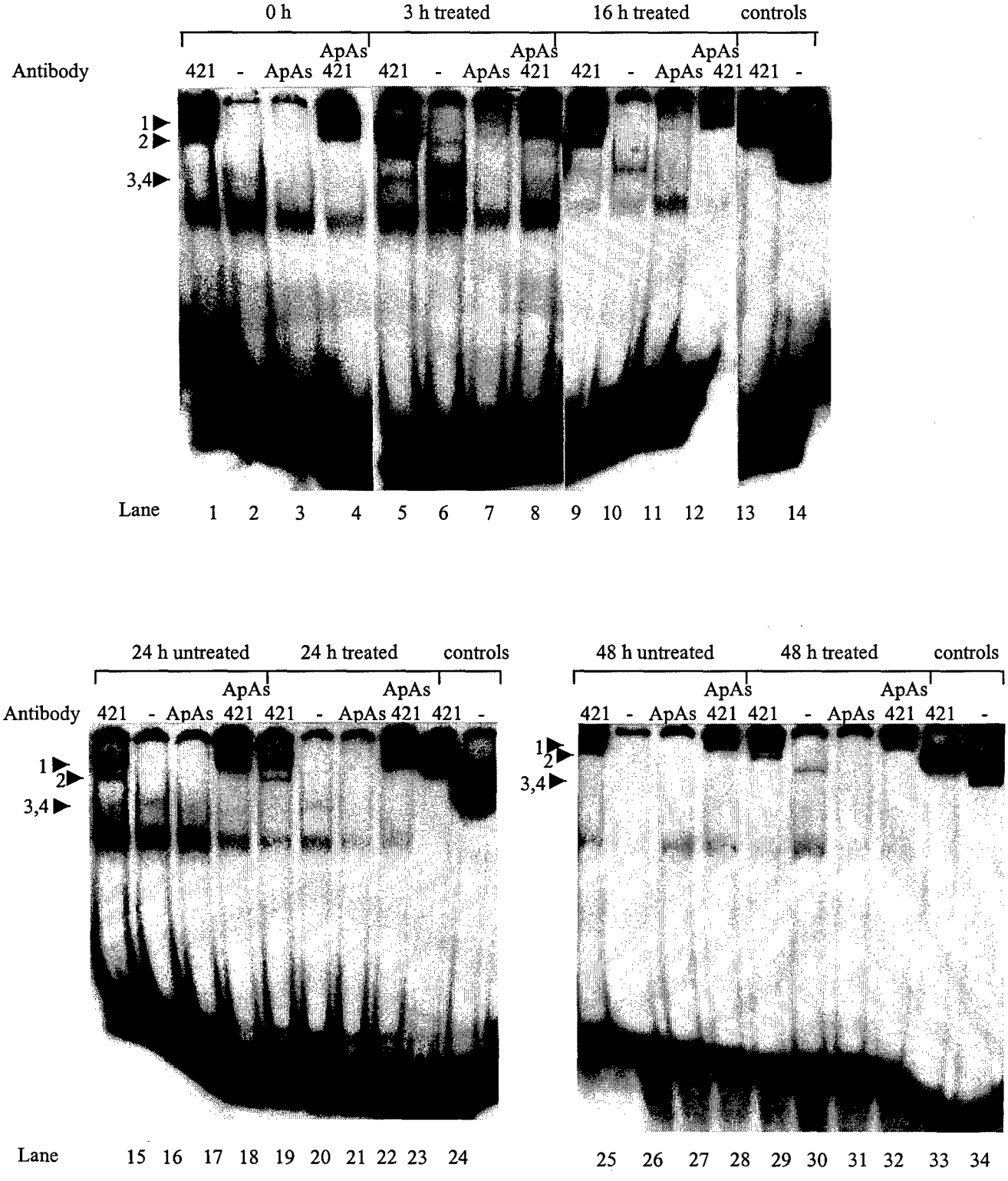



Figure 1. Electrophoretic mobility shift assays of TM10 cell lysates. Binding complexes are indicated: form 1, latent p53 homo-oligomer; form 2, latent p53/p53as hetero-oligomer; form 3, active p53as homo-oligomer; form 4, active p53/p53as hetero-oligomer. Lysates were treated with either 20 μM genistein (treated) or DMSO (untreated). Control reactions contain purified histidine-tagged baculovirus-produced p53 or p53as recombinant proteins. Free probe is at the bottom of the autoradiogram. Treatment of cells was carried out at least twice and electrophoretic mobility shift assays from each set of lysates was analyzed two times.

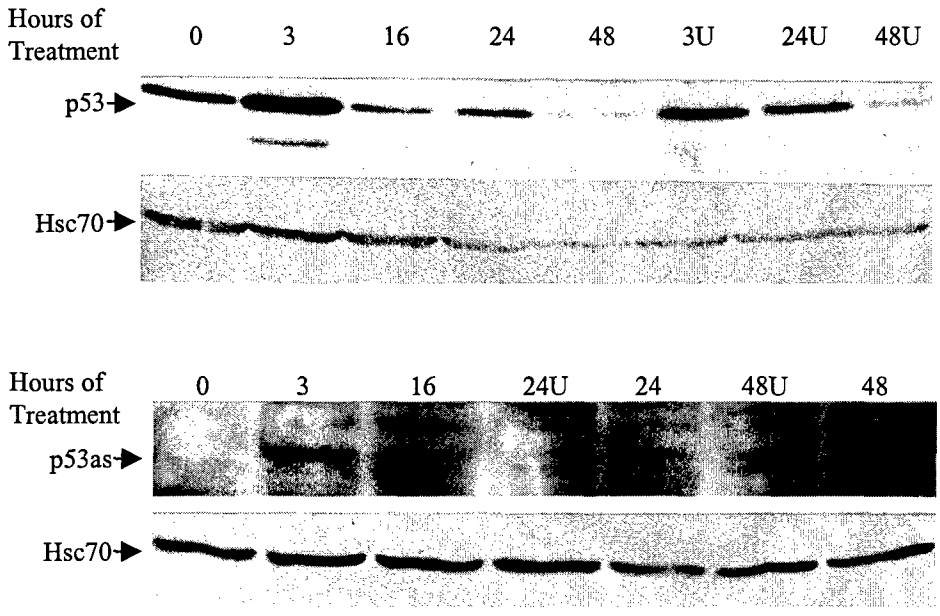



Figure 2. Western immunoblot analyses of genistein-treated TM10 cell lysates. The hours of genistein (20 μM) or DMSO solvent control (U) treatments are indicated. Immunoblots using antibody against heat shock protein (Hsc70) are shown as loading controls. Densitometric quantitation of this autoradiogram was carried out and values for p53 or p53as were normalized to hsc70 to insure equal amounts of proteins were analyzed. Treatment of cells was carried out at least twice and immunoblots from each set of lysates analyzed two times.

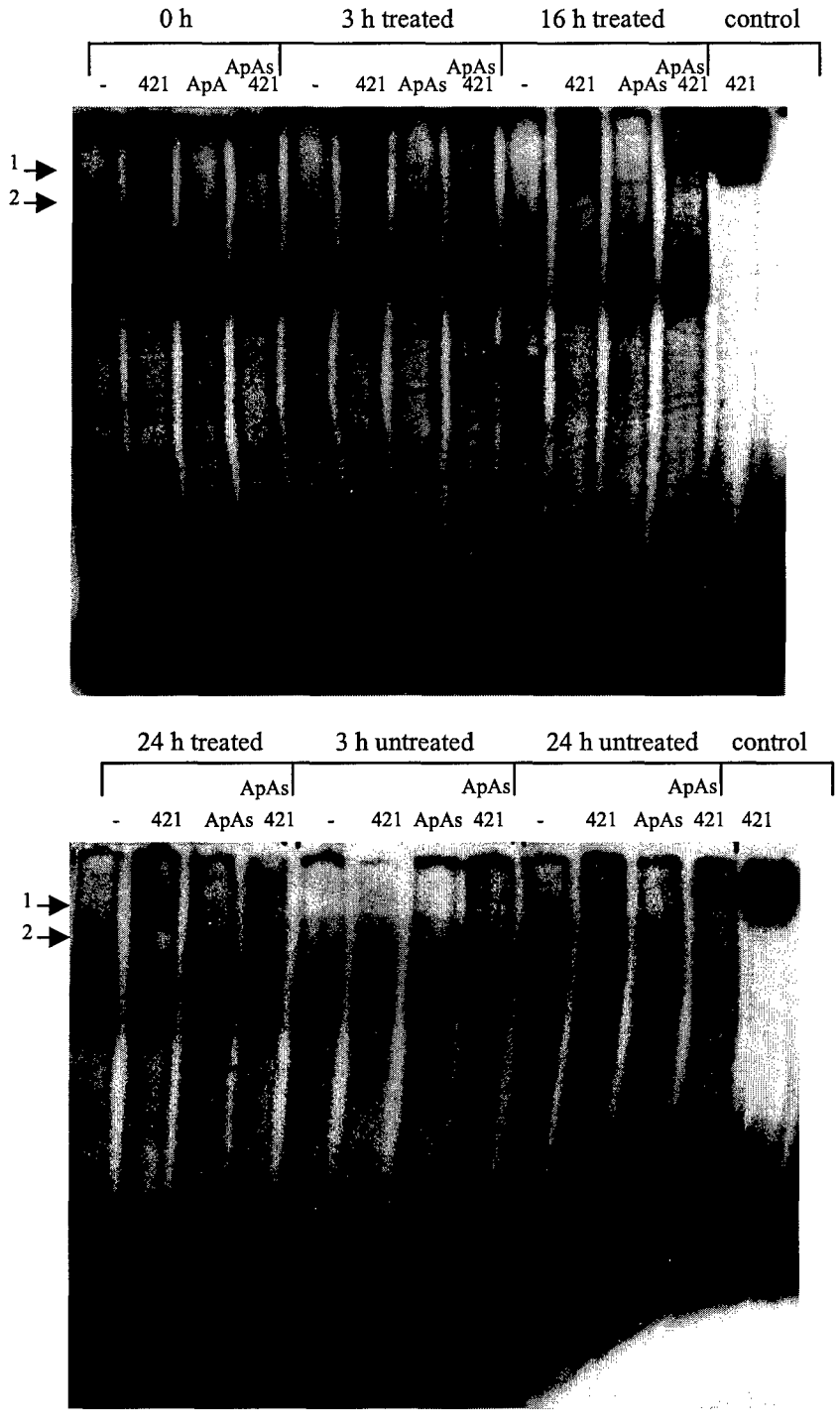



Figure 3. Electrophoretic mobility shift assays of TM12 cell lysates following treatment with genistein. Binding complexes are indicated: form 1, latent p53 homo-oligomer; form 2, latent p53/p53as hetero-oligomer. Cells were treated with either 20 μM genistein (treated) or DMSO (untreated). Control reactions contain purified histidine-tagged baculovirus-produced p53 or p53as recombinant protein. Free probe is at the bottom of the autoradiogram. Treatment of cells was carried out at least twice and electrophoretic mobility shift assays from each set of lysates was analyzed two times.

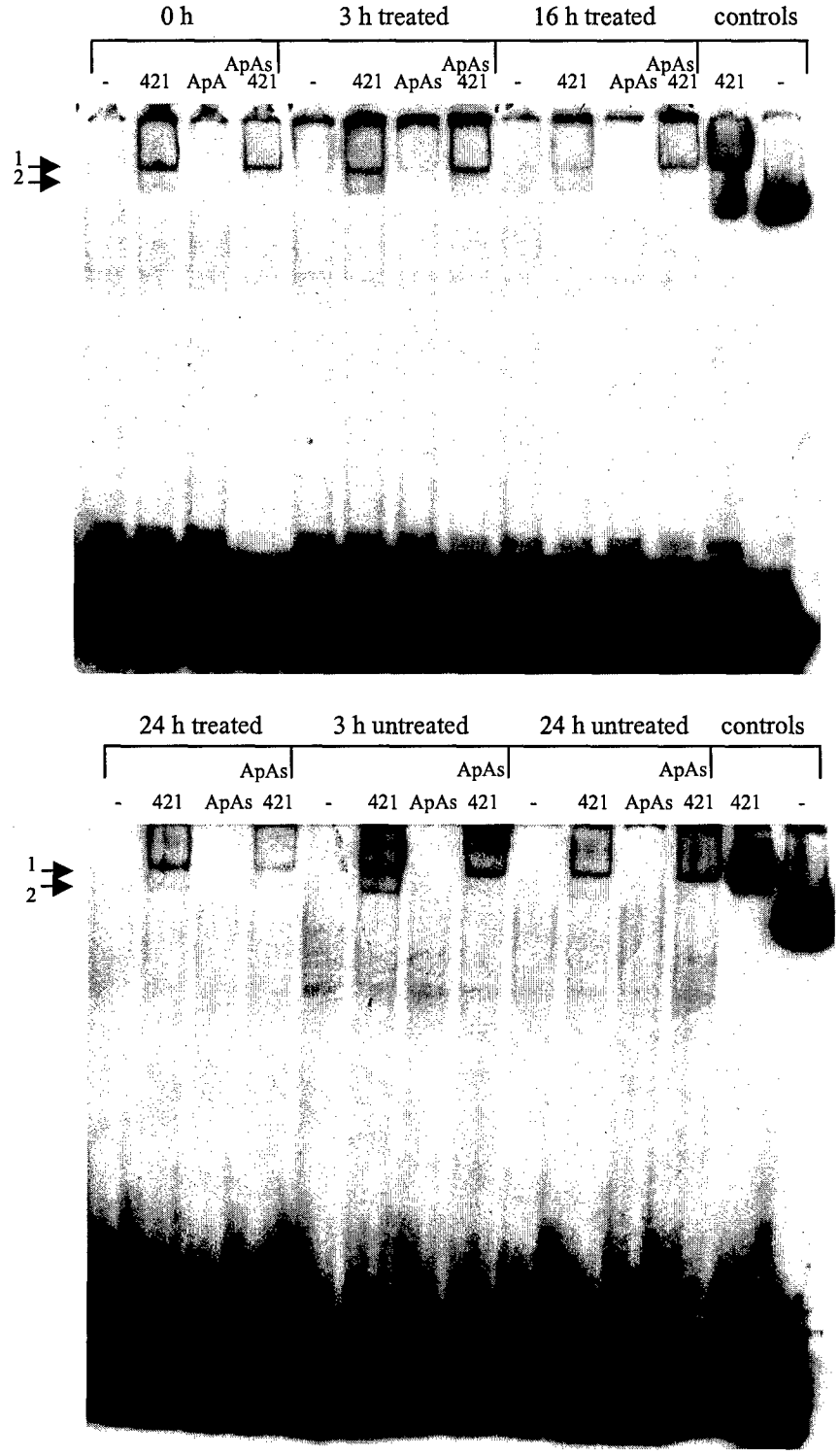



Figure 4. Electrophoretic mobility shift assays of TM10 cell lysates following treatment with X-ray. Binding complexes are indicated: form 1, latent p53 homo-oligomer; form 2, latent p53/p53as hetero-oligomer. Cells were treated with either 400 rads of X-ray (treated) or untreated. Control reactions contain purified histidine-tagged baculovirus-produced p53 or p53as recombinant protein. Free probe is at the bottom of the autoradiogram. Treatment of cells was carried out at least twice and electrophoretic mobility shift assays from each set of lysates was analyzed two times.

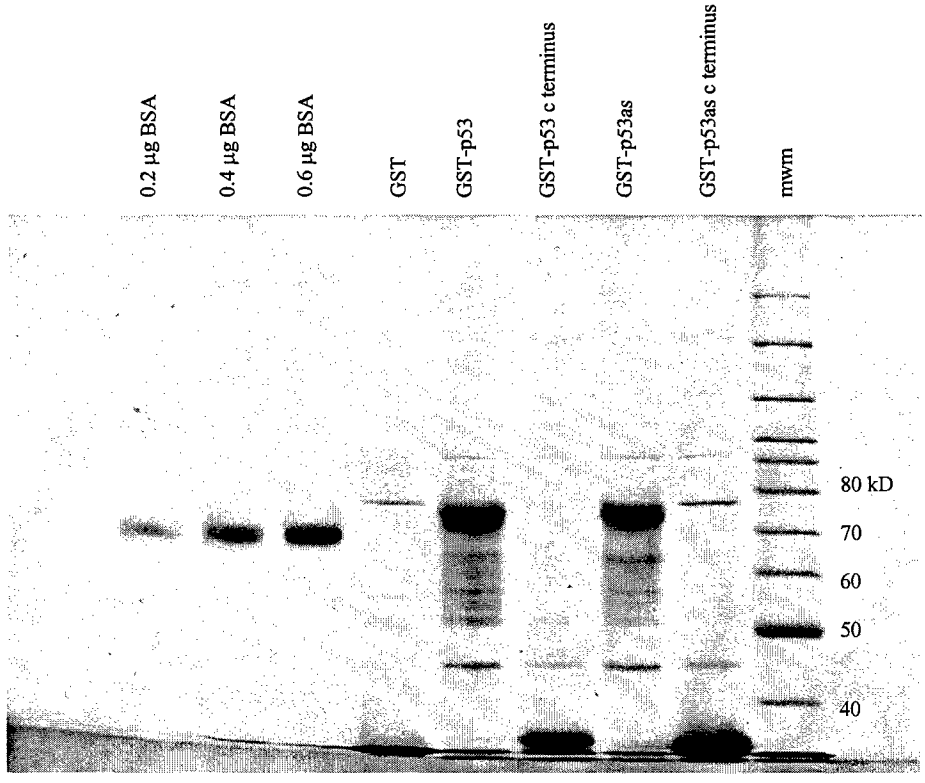



Figure 5. Coomassie Blue stained 7.5% polyacrylamide gel containing GST-fusion proteins. GST, by itself migrates between 25 and 30 kD and is seen at the dye front. Both p53 and p53as c termini proteins contain <100 amino acids fused to GST. p53 and p53as GST-fusion proteins migrate slightly higher than BSA. Molecular weight standards are shown on the right.

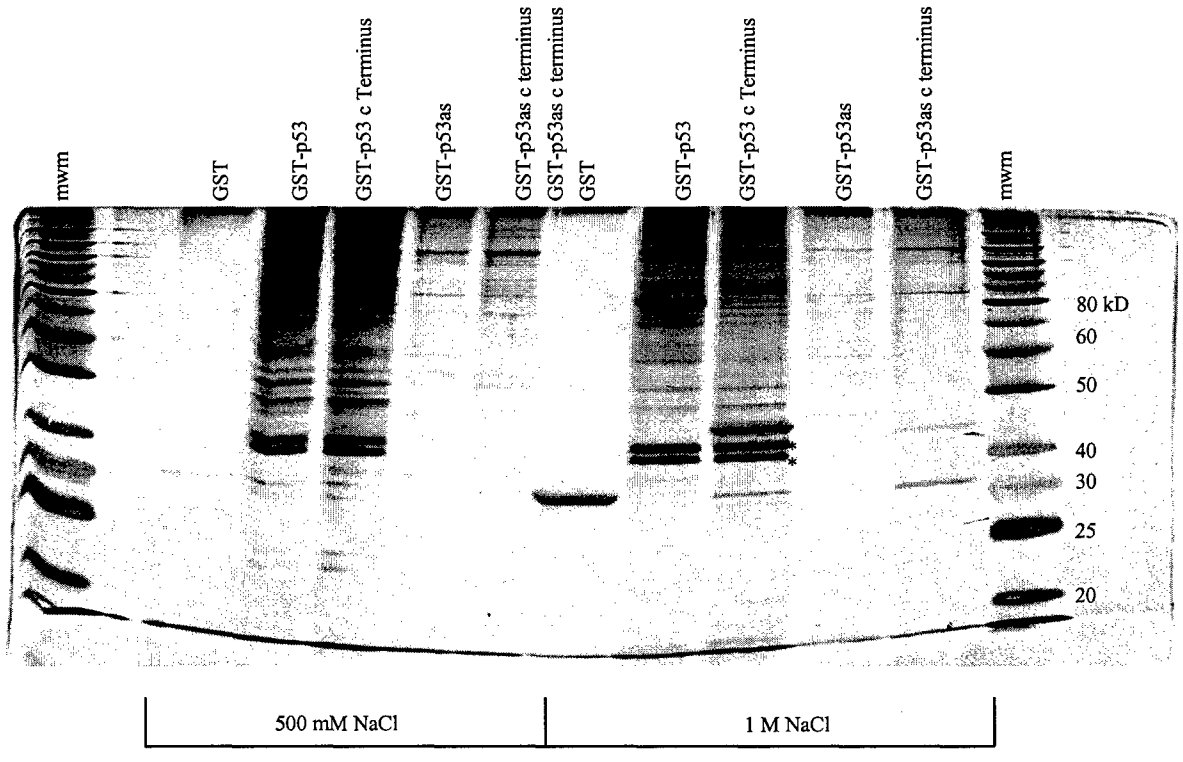
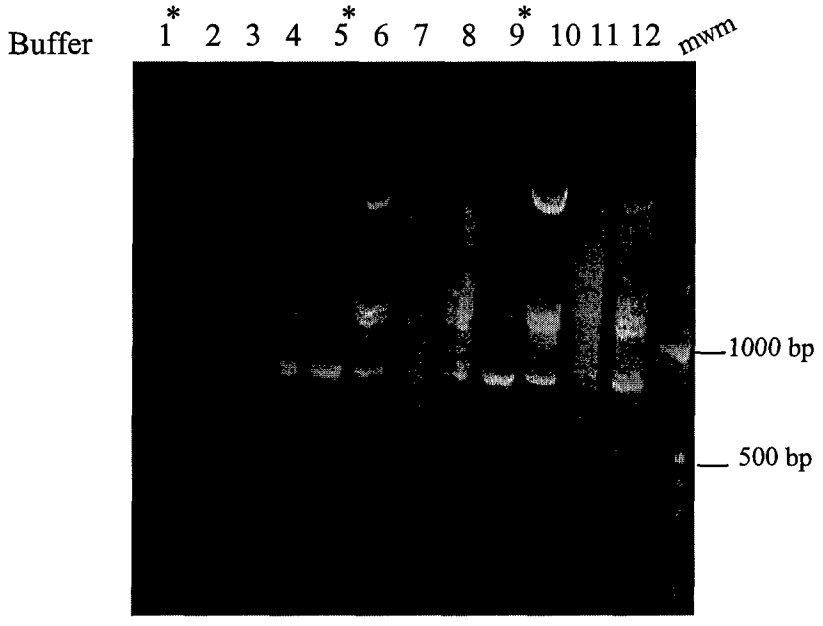



Figure 6. SDS-PAGE resolution of 500 mM and 1 M NaCl fractions of proteins associated with GST-p53 fusion proteins. Two proteins present in p53 and not in p53as are indicated by asterisks. Molecular weight standards are shown on the right.

A




B

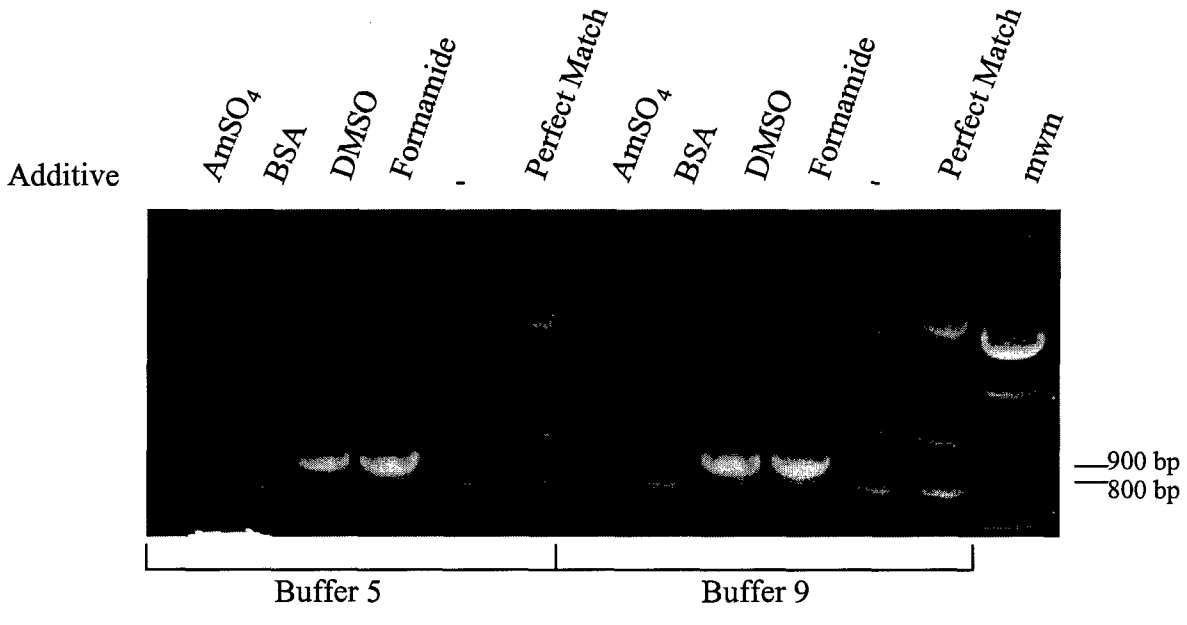



Figure 7. Agarose gel electrophoresis of optimized CArG PCR products. A. Twelve different PCR buffers (Stratagene) were used in individual PCR reactions. Reactions with asterisks gave best product yield with least background. The CArG PCR product is 885 bp. B. Buffers 5 and 9 were used for further analysis with PCR additives as indicated: $AmSO_4$, ammonium sulfate; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; -, no additive; Perfect Match (Stratagene); mwm, molecular weight marker.

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